A MICROBIOLOGICAL DETERMINATION OF THE AMINO ACIDS IN SORGHUM GRAINS AND THEIR MILLED FRACTIONS

by

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INTRODUCTION AND REVIEW OF LITERATURE

Due to the fact that sorghum grains are more adaptable to the prevailing growth conditions in Kansas than corn, they are of value as animal feed and also food for humans. Research has shown that sorghum grain starch is superior to corn starch in many respects in the preparation of food. The ability of the sorghum starch to compete with corn starch depends upon the successful utilization of the milled by-products. The principal outlet for these milling by-products would be largely in the preparation of poultry and livestock feeds.

Utilization of plant protein in animal feed may lead to a deficiency state because the biological value of plant protein may not be sufficient to maintain growth. Absence or suboptimal levels of one or more essential amino acids would result in limited growth of an animal. Consequently, in order to make proper utilization of milling by-products of sorghums, something of the composition of these products should be known. The essential amino acids most likely to be deficient in plant protein are methionine, tryptophane, and lysine.

To date there is very little information on the amino acid content of the sorghum grains or their mill fractions. Lyman, Mosley, Butler, Wood, and Hale (1946) report that the methionine content of whole grain kafir is 0.15 per cent. Almquist (1948) gives milo as containing 0.15 per cent methionine, 0.03 per cent tryptophane, and 0.25 per cent lysine.

There are three recognized methods of determining amino acid deficiencies. These methods are biological, microbiological, and chemical. A biological assay employs the use of laboratory animals such as rats, guinea pigs, or chickens and is based upon the amount of assay material required for satisfactory growth. Chemical and microbiological assays are accurate quantitative methods of determining the amount of assay material present in a given sample.

The purpose of this investigation was to determine the limitations of sorghum grain and its mill fractions for use as complete or partial replacement of corn or wheat products in animal feeds. The essential amino acids methionine, tryptophane, and lysine in the whole grains and milled by-products were determined by the microbiological assay method. It was chosen as the research tool over the other methods because of its distinct advantages of time, size of sample, and expense of operation. Since cystine bears a relation to the amount of methionine that must be supplied, it also was determined on the products from one variety of sorghum grain.

Microbiological methods, based upon the production of lactic acid by the growth of the microorganisms, have been developed by Dunn et al. (1944, pp. 703-713 and pp. 715-724), Stokes et al. (1945), Reisen et al. (1946), Lyman, Mosley, Wood, and Hale (1946), and others.

EXPERIMENTAL

Two varieties of sorghum grain were chosen for analysis, Waxy milo or "Double-Dwarf Waxy Sooner" S. A. 5674-12-5-1 and Westland, Kansas No. 149. Bran, bran fines, grits, mill fines, and germ fractions were obtained on milling. Bran is the outermost seedcoat layer while bran fines are obtained from the finer undercoating. The grits are the broken endosperm fraction of the seed and mill fines are a grit screening fraction. The germ fraction is the seed embryo. Table 1 shows the approximate per cent of each milled fraction from the whole grain.

Table 1. Approximate per cent of each milled fraction from the whole grain.

Sample	: Waxy milo 192-2	: Westland 119-4
Bran fines	2.4	3.1
Bran	7.3	6.1
Mill fines	11.9	5.9
Grits	54.0	71.1
Germ	19.1	15.1

The samples of each mill fraction, including the whole grain, were ground in a Wiley mill through a 20-mesh screen, in preparation for analysis.

In order to have some idea of the quantity of sample to use for microbiological analysis, each was analyzed for total protein. The procedure for this was the A. O. A. C. Official Kjeldahl method.

The principle of the microbiological method of analysis is to measure the growth response of bacteria, yeasts, or molds to graded increments of the amino acid or substance to be determined and of a properly prepared sample. The growth medium is complete except for the nutrient under assay. The amount of growth is determined by measuring either turbidity or lactic acid formed. The latter is more commonly used. The standard curve is plotted and the amount in the sample determined by reference to the curve.

The samples for the determination of methionine, lysine, and cystine were prepared by hydrolyzing one gram samples with 20 ml of 2 N hydrochloric acid for eight hours in an autoclave at 15 pounds pressure. The hydrolysates were cooled, filtered, adjusted to pH 6.8 with sodium hydroxide, and diluted to 100 ml or a convenient volume.

Tryptophane assay was made from samples that had been hydrolyzed with barium hydroxide. This method was a variation of that developed by Greene and Black (1944). A one gram sample was mixed with one gram of barium hydroxide and 20 ml of water and hydrolyzed in an autoclave for eight hours at 15 pounds pressure. Following hydrolysis, the barium hydroxide was removed by lowering the pH to 4 with dilute sulfuric acid, heating to boiling, and filtering. The filtrate, containing the amino acids of the hydrolysate was then extracted three times with 25 ml portions of diethyl ether. The pH was readjusted to 6.8 and the volume diluted to 100 ml. Since racemization of the tryptophane occurs during alkaline hydrolysis, it is necessary to apply a factor of

two in the calculations because only the l-isomer is utilized by the bacteria.

For the determination, the hydrolysates were added to the assay tubes of basal media at four or five different levels. After the tubes had been sterilized, they were inoculated with 'Leuconostoc mesenteroides' and incubated for a period of 48 to 72 hours. A set of tubes containing gradient amounts of the amino acid under analysis were treated similarly. The lactic acid produced by the microorganisms is proportional to the growth (multiplication) of the organism which, in turn, is directly related to the amount of amino acid under assay. The lactic acid present was measured by titration with a 0.05 N sodium hydroxide solution. The titration values of the standard tubes were plotted against the concentration of the amino acid, under assay, and a standard curve obtained. The quantity of amino acid present in each graded aliquot of a sample was determined by reading the amount of amino acid corresponding to the titration value from the standard curve.

Each assay was carried out in duplicate sets. Standard curves for L-methionine, L-tryptophane, L-lysine, and L-cystine ranged from 0-45, 0-10, 0-200, and 0-100 micrograms, respectively, of the amino acid.

The acid and alkaline hydrolysates contained one gram of sample for each 100 ml of solution. In the assay procedure, the following aliquots of the acid hydrolysate were used: for methionine, the equivalent of 5-20 mg of the original sample, except for the germ fraction which was 5-8 mg; for lysine 5-25 mg of

sample for all fractions except the germ fraction which was 4-20 mg; and for cystine 5-25 mg of sample for all fractions except the germ which was 4-20 mg. The aliquots for tryptophane assay were taken from the alkaline hydrolysates and ranged from 8-20 mg of the original sample.

The amino acids not under assay were supplied in sufficient amount in the basal medium to permit maximum growth. They were supplied by one of two different methods, as crystalline amino acids in the determination of lysine, and for methionine, cystine, and tryptophane assays in the form of a hydrogen peroxide treated peptone solution.

A stock of this peroxide treated peptone was prepared according to Lyman et al. (1946) and kept for considerable time under sterile conditions. The preparation is as follows: Dissolve 50 grams of peptone in 250 ml of water. When solution has taken place, add 250 ml of 2 N hydrochloric acid and about 3 ml of 30 per cent hydrogen peroxide and allow to stand over night at room temperature. The solution is then steamed for 30 minutes with periodic shaking, cooled, and neutralized with sodium hydroxide. A second steaming for one hour is now necessary to destroy any remaining hydrogen peroxide not used up in the oxidative process. The pH should be adjusted to 6.8 and the volume made up to one liter. The peptone solution is now ready for use but should be stored under sterile conditions in a refrigerator. This treatment with hydrogen peroxide destroys the methionine, tryptophane, cystine, and tyrosine. The solution may be used for the determina-

tion of any of these four amino acids by adding the other three to the basic media.

The ingredients for the complete basal medium are found in Tables 2 and 3. Table 2 is the medium adapted for lysine assays and involves the use of individual amino acids, similar to media used by Dunn, Shankman, Camien, Frankl, and Rockland (1944).

Table 2. Composition of basal medium for lysine determinations (100 tubes).

Nutrient	Amount	Nutrient	Amount
DL-alanine	2000 mg	glucose	20 g
L(+)-arginine HCL	80	adenine sulphate	12 mg
asparagine (natural)	400	guanine HCl	12
L(-)-cystine	120	uracil	12
L(+)-glutamic acid	150	sodium acetate	12 g
glycine	100	NH_4Cl	6
L(-)-histidine $HCl.H_2O$	20	KH_2PO_4	500 mg
L(-)-hydroxyproline	100	K_2HPO_4	500
DL-isoleucine	150	$MgSO_4.7H_2O$	200
L(-)-leucine	75	NaCl	10
DL-methionine	40	$FeSO_4 \cdot 6H_2O$	10
DL-norleucine	100	$MnSO_4 \cdot 4H_2O$	10
DL-norvaline	100	thiamine HCl	1
DL-phenylalanine	60	pyridoxine	1.6
L(-)-proline	25	DL-Ca pantothenate	2
DL-serine	80	riboflavin	2
DL-threonine	450	nicotinic acid	2
L(-)-tryptophane	10	biotin	5
L(-)-tyrosine	30	folic acid	2
DL-valine	150	p-aminobenzoic acid	0.1

Table 3 is the peptone medium adapted from Lyman, Mosley, Butler, Wood, and Hale (1946). Vitamin concentrations were increased somewhat and the acetate buffer was also increased.

The organism chosen for these analyses was 'Leuconostoc mesenteroides', one of the lactic acid producing microorganisms. This

organism was chosen because of its rapid growth, stability to higher salt concentrations, and its requirement of all four of the amino acids analyzed for.

Table 3. Peptone medium for Leuconostoc mesenteroides (100 tubes).

Nutrient	Amount	Nutrient	Amount
H_2O_2 treated peptone[1] (150 ml)	7.5 g	adenine sulphate	0.1 mg
glucose	20	guanine HCl	0.1
sodium acetate	20	uracil	0.1
ammonium chloride	6	xanthine	10
L-tyrosine	50 mg	riboflavin	2
L(-)-cystine[2]	100	thiamin HC_1	1
DL-tryptophan[2]	100	niacin	2
DL-methionine[2]	200	pyridoxine	2
KH_2PO_4	0.5 g	Ca-pantothenate	2
K_2HPO_4	0.5	p-aminobenzoic acid	0.2
$MgSO_4 \cdot 7H_2O$	0.2	biotin	5 mcg
NaCl	0.01	folic acid	10
$FeSO_4 \cdot 7H_2O$	0.01	pyridoxamine di HCl	0.5 mg
$MnSO_4 \cdot 4H_2O$	0.01		

(1) Replaces the individual amino acids in Table 2.

(2) The amino acid to be determined is left out.

The organism was maintained on stabs of tomato juice agar and kept in a refrigerator after 24 hours growth. Transfers were made at four week intervals, or more often if necessary, to maintain a pure and active strain.

Preparation of the inoculum was made by transferring some of a stab growth to a liquid peptone medium and incubating for 24 hours. The organisms in the incubated liquid medium were then centrifuged and the supernatant liquid poured off. They were then washed and centrifuged three times with a sterile isotonic saline

solution to remove any of the amino acid under assay that may remain from the peptone media on which the organism was grown. The organisms were suspended in 20 ml of the isotonic saline solution in readiness for inoculation of the assay tubes.

Typical standard curves obtained are shown in Figs. 1, 2, 3, and 4.

DISCUSSION

The results of the amino acids analyses are shown in Table 4. They represent an average of three or more analyses run in duplicate sets with each sample carried at four or five different levels. The cystine values were obtained from a single analysis run in duplicate. The second whole grain and its mill fractions were not analyzed for cystine because only an indication of its content was desired and analysis of one set would give this information.

The protein content of Waxy milo and its milled products, bran fines, grits, and germ, are noticeably higher than Westland for the sample analyzed. The bran and mill fines are only slightly higher. The bran fines and grits bear about the same relation to their corresponding whole grains in both cases.

The Waxy milo sample was slightly lower in protein than corn while the Westland was much lower. Thus, both have a definitely lower protein value than wheat. From information available, the protein content of corn and wheat by-products vary in the same proportion to the whole grain as do the sorghums.

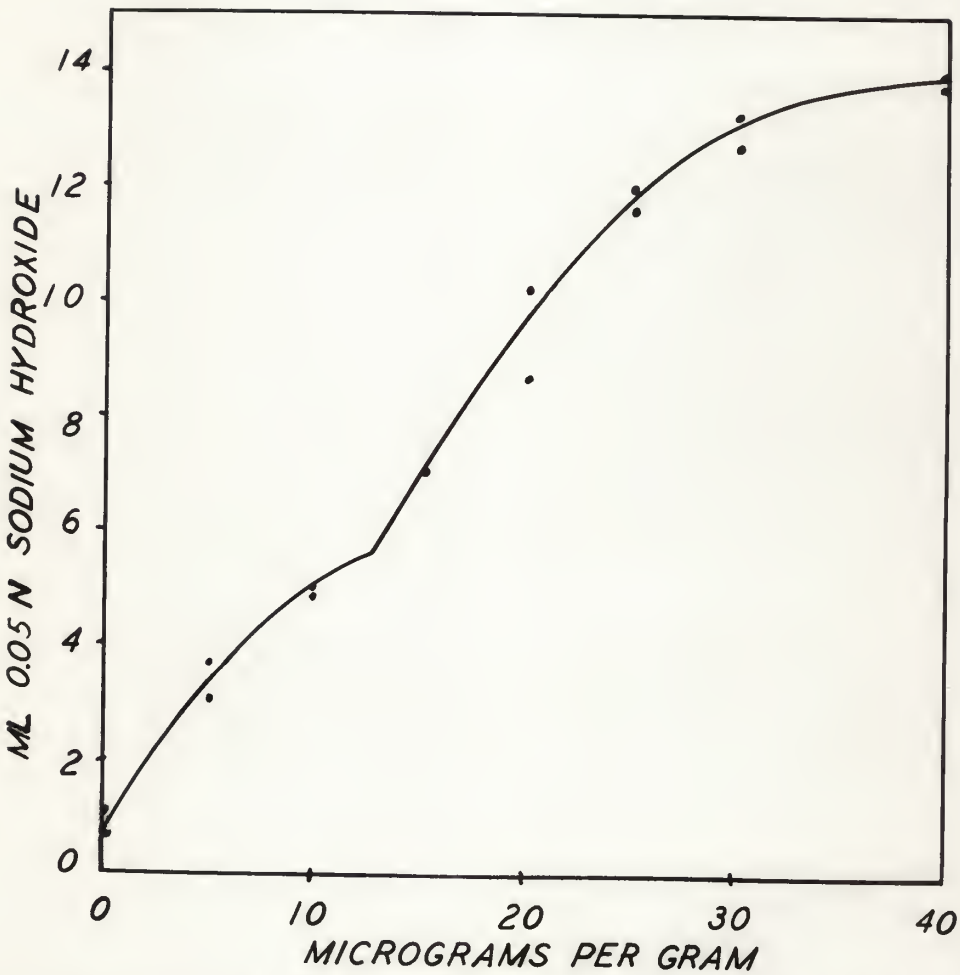

FIG. I. TYPICAL STANDARD CURVE FOR THE DETERMINATION OF L-METHIONINE WITH LEUCONOSTOC MESENTEROIDES.

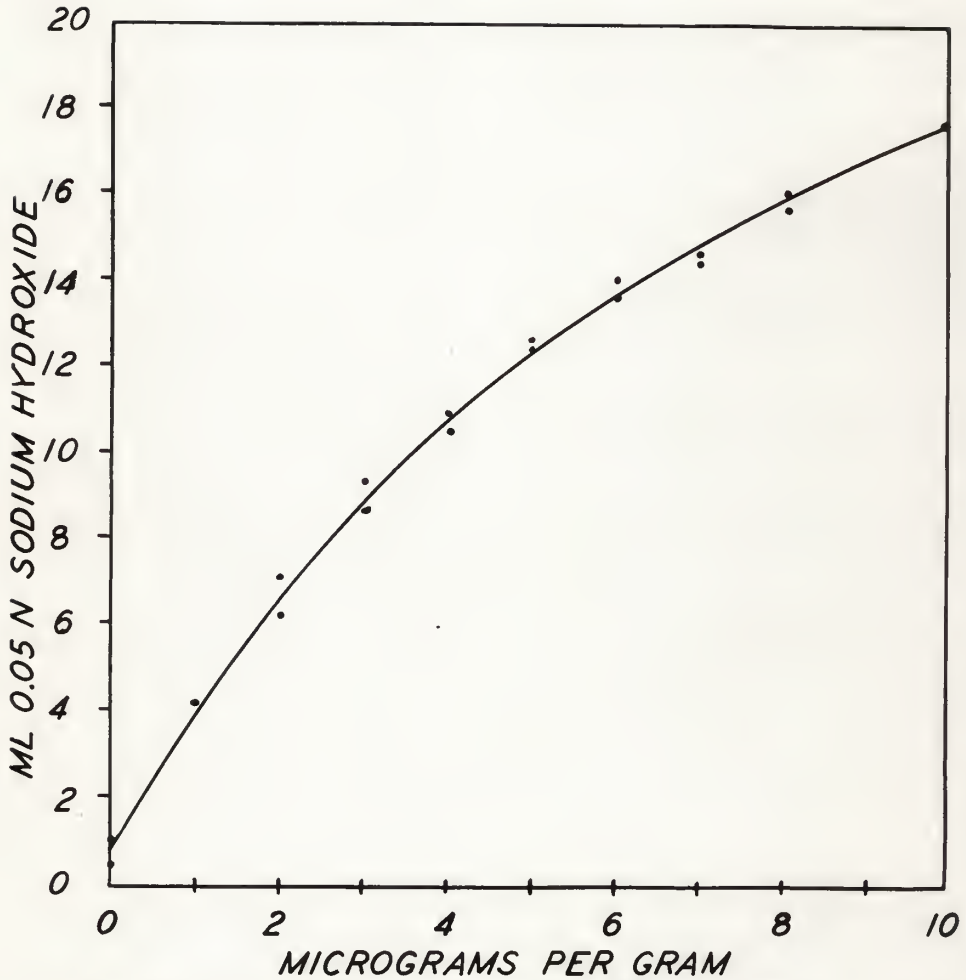

FIG. 2. TYPICAL STANDARD CURVE FOR THE DETERMINATION OF L-TRYPTOPHANE WITH LEUCONOSTOC MESENTEROIDES.

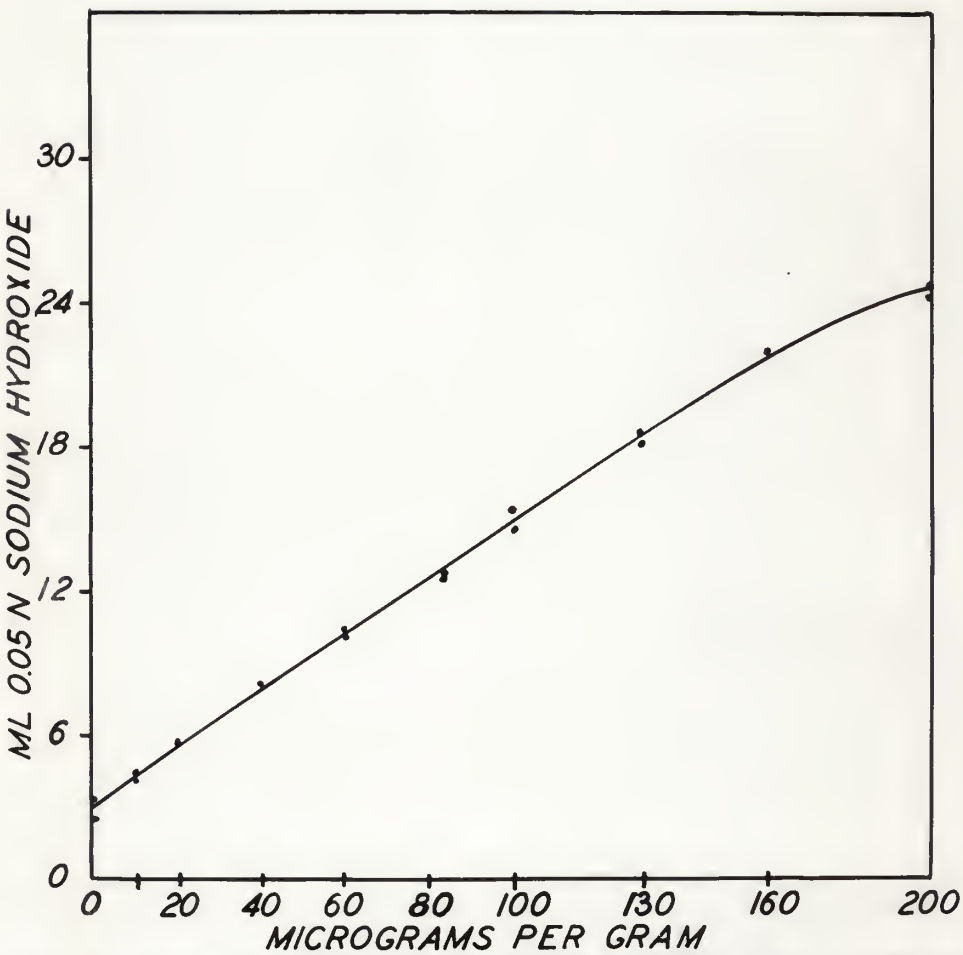

FIG. 3. TYPICAL STANDARD CURVE FOR THE DETERMINATION OF L-LYSINE WITH LEUCONOSTOC MESENTEROIDES.

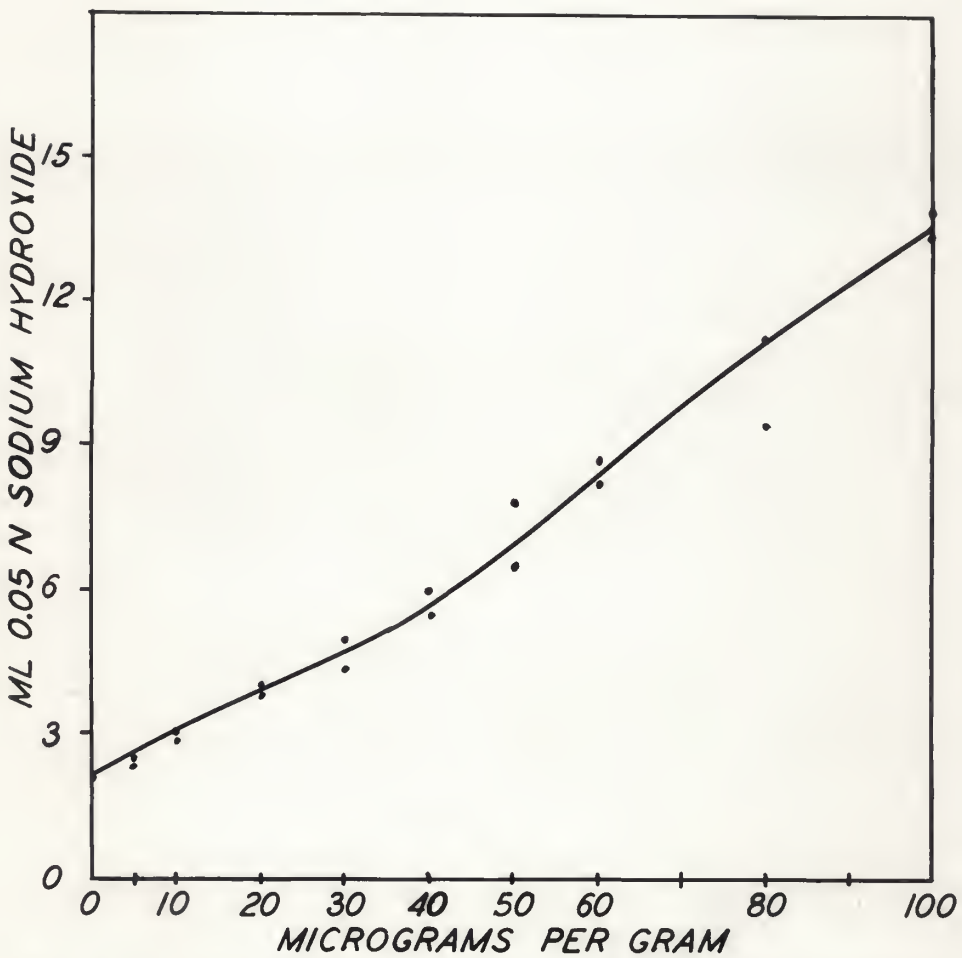

FIG. 4. TYPICAL STANDARD CURVE FOR THE DETERMINATION OF L-CYSTINE WITH LEUCONOSTOC MESENTEROIDES.

Table 4. Per cent of amino acids in samples.

Sample	Per cent protein	Methionine	Tryptophane	Lysine	Cystine
Waxy Milo (192-2) Double-Dwarf Waxy Sooner S.A. 5674-12-5-1					
Whole grain	9.01	.165	.056	.326	.082
Bran fines	6.72	.123	.052	.294	.134
Bran	4.84	.081	.018	.208	.116
Mill fines	6.62	.122	.044	.222	.175
Grits	8.73	.135	.050	.149	.078
Germ	12.42	.253	.082	.731	.255
Westland (119-4) Kansas No. 149					
Whole grain	7.48	.133	.029	.251	
Bran fines	5.43	.097	.011	.220	
Bran	4.60	.078	.026	.193	
Mill fines	6.44	.123	.028	.191	
Grits	7.19	.151	.025	.088	
Germ	11.70	.240	.060	.732	

On a protein basis the tryptophane content of the Westland and its mill fractions are much lower than the Waxy except for the bran fraction, Table 5. According to the analysis, the tryptophane content of the bran fraction is much higher in the Westland variety than the Waxy.

In a similar manner there is little difference between the methionine content of the two whole grains or their milling by-products. The single exception was the Westland grits which were much higher than the corresponding Waxy fraction or the Westland whole grain and as high as the germ. The lysine content of this same Westland fraction was about one-third lower than the Waxy grits with all other fractions agreeing with the corresponding ones of Waxy milo. Apparently there is a difference in amino acid

Table 5. Per cent of amino acids in the proteins.

Sample	Methionine	Tryptophane	Lysine	Cystine
Waxy Milo (192-2) Double-Dwarf Waxy Sooner S.A. 5674-12-5-1				
Whole grain	1.83	0.62	3.62	0.91
Bran fines	1.83	0.80	4.37	1.99
Bran	1.67	0.37	4.30	2.40
Mill fines	1.81	0.67	3.36	2.64
Grits	1.54	0.57	1.70	0.89
Germ	2.04	0.66	5.90	2.03
Westland (119-4) Kansas No. 149				
Whole grain	1.77	0.39	3.36	
Bran fines	1.78	0.20	4.05	
Bran	1.69	0.57	4.20	
Mill fines	1.91	0.44	2.96	
Grits	2.10	0.35	1.22	
Germ	2.05	0.51	6.26	

composition of the protein from one variety to another. This is shown by the difference in amino acid content of some corresponding fractions. For example, the protein of the Westland grits is higher in methionine and lower in tryptophane and lysine than the Waxy.

Tryptophane content of the sorghum grain proteins is slightly lower than corn and much lower than the wheat values, Table 6.

Methionine is slightly lower in the sorghum grains analyzed than in wheat and lower than in corn. On the other hand, as shown in Table 6, the lysine content of whole grain sorghums and their milled by-products are somewhat higher than corresponding corn or wheat products.

Table 6. Per cent of amino acids in the cereal proteins.

Grain fraction	Methionine	Tryptophane	Lysine	Cystine
Wheat (1)	2.0	1.2	2.7	1.3 ± 0.3
Wheat (2)	1.3	1.7	2.8	1.8
Wheat bran (2)	1.8	1.5	3.3	1.6
Wheat germ (1)	2.0	1.0	6.4	0.6
Corn (1)	2.7	0.8	2.0	1.1
Corn (2)	2.7	0.7	2.2	1.9
Corn germ (1)	2.3	1.3	5.8	1.2 ± 0.3
Milo (2)	1.5	0.3	2.5	2.0

(1) Block and Bolling (1945) chemical method of analysis

(2) Almquist (1948).

The whole grain, bran, and germ fraction of the sorghums are higher in lysine content than the corresponding fractions of corn and wheat, except for wheat germ.

The lysine content of sorghum is sufficient to meet the daily dietary requirements of the chick as the sole source of protein, at 20 per cent protein level. However, in order to use the sorghums or milled by-products successfully, they would naturally have to be fortified with another protein material which has a higher percentage of certain amino acids, methionine and tryptophane especially.

The sorghums may be used in the same way as corn which also requires amino acid supplementation. The sorghum products may be used in place of the corresponding wheat products with some reservation because of lower tryptophane content. It may take slightly more of the supplementary protein to bring the tryptophane and possibly the methionine up to desired level when sorghum product

is used. The necessary methionine may be supplied as crystalline amino acid but because of expense the use of other crystalline amino acids is usually not practical.

There are indications that sorghums have a rather high concentration of some of the B-complex vitamins. The reports of Tanner et al. (1947) indicate riboflavin values of sorghum to be slightly higher than yellow hybrid corn. Sorghums may contain two and one-half to three times as much biotin as corn and are also notably higher in niacin.

Further investigation of different samples of the two sorghums contained in this study is advisable since there may be some variation. Soil and climatic conditions have a great influence upon the composition of the grain. Further analysis for niacin, riboflavin, thiamin, and pantothenic acid should be made before making final conclusion upon how to most successfully use the sorghums in feeds.

SUMMARY

The microbiological assay method was used for the determination of methionine, tryptophane, and lysine using 'Leuconostoc mesenteroides' as a test microorganism. Two varieties (a waxy and a non-waxy) of sorghum were analyzed. It was found that the sorghums are lower than corn and wheat in methionine and much lower in tryptophane than wheat. However, lysine content is much higher in the sorghums than it is in either corn or wheat.

It is possible to use the sorghums in place of the correspond-

ing corn or wheat product with certain reservations. Further investigation of more samples and other varieties would be advisable. Also, analysis of some of the B-complex vitamins in sorghums would be helpful in making conclusions as to how they could be properly used in feeds.

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